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EMBRYONIC DEVELOPMENT OF THE BRITTLE-STAR *AMPHIPHOLIS KOCHII* IN LABORATORY CULTURE

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Abstract

The embryonic development of the brittle-star *Amphipholis kochii*, from fertilization through metamorphosis, was observed in a laboratory culture. Oocytes from spawning induced by a sudden change of sea water temperature remain in the first meiotic metaphase until fertilization. The unfertilized egg, about 90 μm in diameter, is opaque, brownish red, and homolecithal. The fertilization membrane of the egg is transparent and non-sticky, and the translucent (7–8 μm) hyaline layer is thick. Cleavage is holoblastic and equal. The blastomeres usually are irregularly arranged, although some eggs show the regular radial cleavage. The archenteron is formed by an invagination of the endodermal cells. The developing larval spicules first take a tetraradiate form, unlike the triradiate forms of most other ophioplutei and echinoplutei. The fully grown ophiopluteus has eight arms and a very simple skeletal system. The left posterior coelom is divided into a hydrocoel and a somatocoel. The anterior part of the larva and the larval arms, except for the postero-lateral arms, shrink and degenerate by the onset of metamorphosis.

Introduction

The ophiuroids have various developmental patterns, and planktotrophic development with a free-living ophiopluteus is typical (Hyman, 1955; Hendler, 1975). However, recent reports on ophiuroid development have been concerned with the pattern known as abbreviated development (Fenaux, 1963, 1969; Patent, 1970; Stancyk, 1973; Hendler, 1977, 1978; Mladenov, 1979; Oguro *et al.*, 1982). Observations made on development of the ophiopluteus larva are scarce, despite the fact that many ophioplutei have been found by means of plankton-hauls (*e.g.*, Mortensen, 1913, 1920, 1921, 1931, 1937, 1938). To date, detailed studies on ophiopluteus development have been confined to the three species *Ophiothrix fragilis* (MacBride, 1907), *Ophiocoma nigra* (Narasimhamurti, 1933), and *Ophiopholis aculeata* (Olsen, 1942). In addition, reports on the early developmental processes of ophiuroids, including fertilization and early cleavage, are very limited, due to the difficulty in artificially inducing spawning in the laboratory. Indeed, no chemical substances have been reported to induce spawning in the ophiuroids, whereas KCl and 1-methyladenine will induce spawning in other echinoderm groups.

Recently, however, the author has developed a method to induce spawning in the brittle-star *Amphipholis kochii* and has confirmed that this species shows a typical ophiuroid developmental pattern with a free-living ophiopluteus. The present paper describes the embryonic development of the brittle-star *Amphipholis kochii*, from fertilization to metamorphosis, in laboratory culture, with special reference to the early developmental processes.

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Materials and Methods

The brittle-star, *Amphipholis kochii*, was found under stones between the tidemarks at Abuta on the Pacific coast of southwestern Hokkaido, Japan. Samples of the mature animals were taken during their breeding season in June and July (Iwata and Yamashita, 1982; Yamashita and Iwata, 1983).

Fertilization of eggs in the laboratory was carried out according to the method previously described (Yamashita, 1983). Embryos and larvae were reared in sea water filtered through a 0.45-μm Millipore filter and treated with antibiotics, penicillin G (100 units/ml), and streptomycin sulfate (1 mg/ml). Artificial sea water (Jamarin U; Jamarin Lab., Osaka, Japan) was also used for the culture medium. The culture medium was gently stirred (*ca.* 60 rpm) as in echinoplutei cultures (Hinegardner, 1969), and was changed daily during early development and every other day during later stages. The temperature of the medium was maintained at 23°C or 15°C. Larvae were fed laboratory cultures of the diatom, *Phaeodactylum tricornutum*.

The embryos and larvae were taken from the culture dish at appropriate intervals and observed under a light microscope. To analyze the cleavage pattern of the early embryos, several fresh samples were also observed by means of a time-lapse cinematograph.

For histological observation, the embryos and larvae were fixed in Bouin's solution for three hours or more, dehydrated with ethanol, and embedded in paraffin. The serial sections (3–5 μm) were stained with Delafield's hematoxylin and eosin.

Results

Gamete shedding and early development

The brittle-star *Amphipholis kochii* is induced to shed gametes by the temperature shock described previously (Yamashita, 1983). Movement of the brittle-star becomes active after the temperature shock, and 30–40 min following the temperature elevation from 4°C to 23°C, the brittle-star sheds all gametes through five-paired bursal slits. Each female (1 mm disk diameter and containing 70–80 ovaries) spawns about 40,000 eggs. When both males and females are kept in a finger bowl and treated with the temperature shock, males usually spawn before the females. This suggests that the shedding of sperm prior to that of eggs may take place in the field. The shedding posture is similar to those found in other ophiuroids; the disk is raised several centimeters above the bottom and contracts vigorously (Fig. 1) (Mortensen, 1920; Olsen, 1942; Woodley, 1975; Hendler, 1977; Mladenov, 1979; Bowmer, 1982; Hendler and Meyer, 1982).

The spawned eggs are at the first meiotic metaphase and remain in that stage until fertilization (Fig. 2A). The living unfertilized egg, about 90 μm in diameter, is opaque, brownish red, and homolecithal (Fig. 2A). It is slightly heavier than sea water. A transparent jelly coat, measuring 5 μm thick, entirely surrounds the egg surface (Fig. 2A). The unfertilized eggs are capable of forming a fertilization membrane for at least 3 hours after spawning, but the ability of the eggs to develop normally diminishes as fertilization is delayed (Fig. 3).

Fertilization occurs at any point on the egg surface. The spermatozoa which fail to contribute to fertilization remain attached to the surface of the jelly coat (Fig. 4). As soon as cortical reaction occurs, the jelly coat overlying the sperm entry site begins to dissolve. It completely disappears from the whole egg surface within a few minutes postinsemination (Fig. 4). When the jelly coat of the unfertilized eggs is removed by acidic sea water (pH = 5.0), these eggs are no longer fertilizable even in normal sea

FIGURE 1. Shedding posture of *Amphipholis kochii* female induced to spawn by temperature shock. Arrowheads indicate spawned eggs. Shedding posture of the male is the same. Scale, 1 cm.

water, suggesting that the jelly coat plays an important role in fertilization. However, that the eggs were irreparably damaged by acidic sea water cannot be ruled out.

The fertilized egg has a transparent fertilization membrane and a translucent, thick (7–8 μm) hyaline layer (Fig. 2B). The fertilization membrane is not sticky. The first and second polar bodies, measuring 3–4 μm in diameter move freely within the perivitelline space (Fig. 2C). Occasionally the first polar body subdivides into two, so that three polar bodies are seen in the perivitelline space.

Changes in the egg morphology before, during, and after fertilization as observed with transmission and scanning electron microscopes have been described elsewhere (Yamashita, 1983, 1984).

Culture of the embryos and larvae was carried out at 23°C and 15°C, since the field temperature during the breeding season of the present species ranges from 15°C to 23°C (*cf.*, Yamashita and Iwata, 1983). The following description of the embryonic development is based on the culture at 23°C. There is no significant difference between the two cultures, except for developmental speed. The rate of development in the two cultures, together with the Q_{10}, is shown in Table I. The failure to achieve metamorphosis at 15°C (Table I) is not an effect of temperature. It is probably due to unsuitable conditions, caused by long-term culture.

Cleavage is holoblastic and equal. Unequal cleavage, as observed in echinoid development, is not found in this species. The first division cuts the egg along the longitudinal axis (Fig. 2D). During and after the second cleavage, arrangement of the blastomeres becomes irregular: in many embryos, the blastomeres lie across each other, somewhat resembling the arrangement of the spiral cleavage (Figs. 2F, H). Some embryos show the regular radial arrangement of the blastomeres at the early cleavage stages (Figs. 2E, G), but in all cases the blastomeres become irregularly arranged during cleavage.

Cinematographic analysis reveals that this irregular arrangement is due to intrinsically irregular cleavage planes of each blastomere (Fig. 5), although it also reveals that in some embryos the blastomeres are rearranged after the regular radial cleavage. In any case, the embryos develop quite normally regardless of blastomere arrangement. Because of irregular cleavage and movable polar bodies, it is very difficult to follow

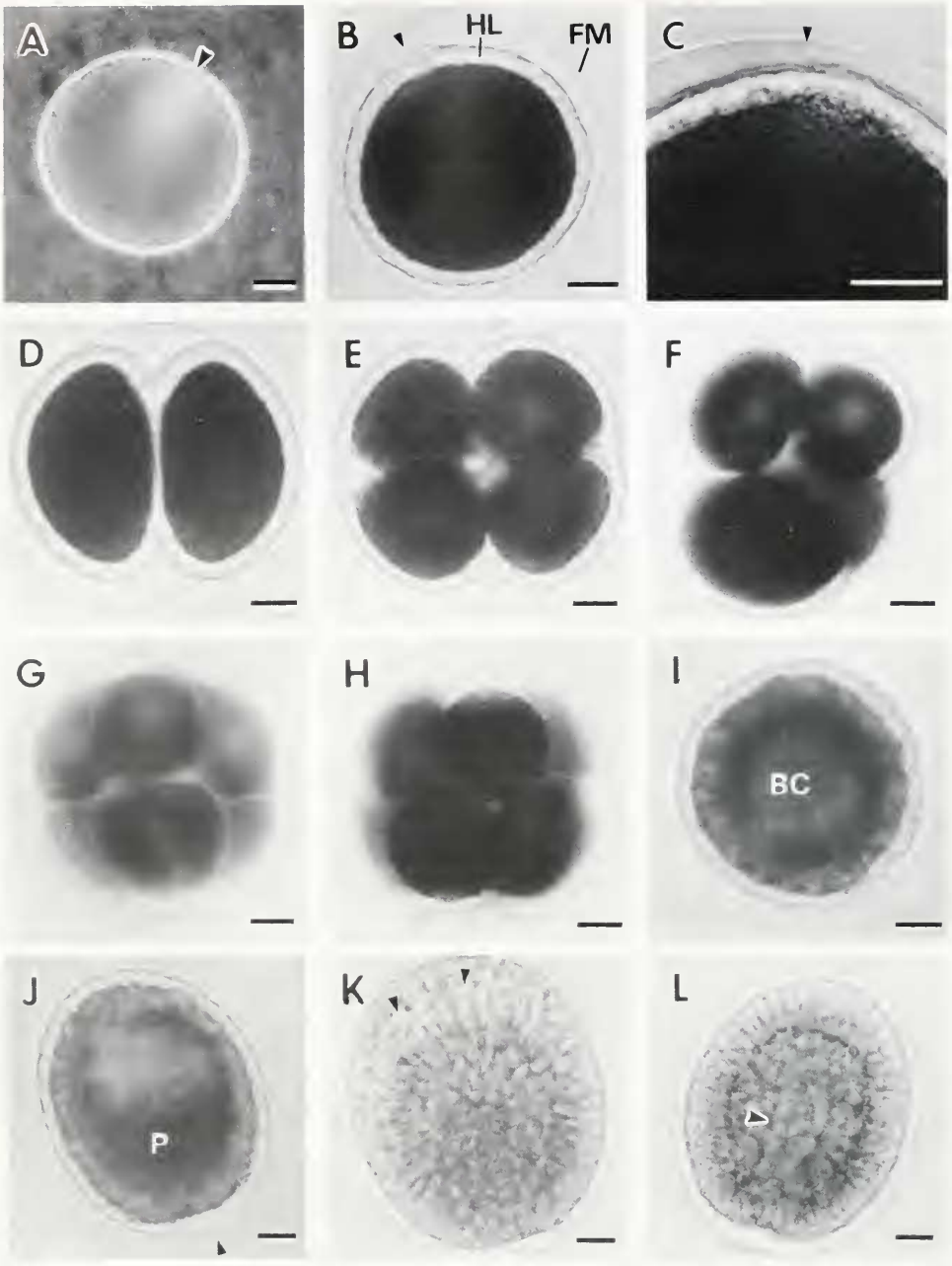

FIGURE 2. Unfertilized egg and early embryos. Scale, 20 μm. A. Unfertilized egg in the sea water containing India ink to show the jelly coat. Arrowhead indicates the region where spindle of the first meiotic metaphase is situated. Lateral view. B. Fertilized egg surrounded by the fertilization membrane (FM) and hyaline layer (HL). Swelling of the hyaline layer at the animal pole (arrowhead) is the elimination site of the first polar body. Lateral view. C. High magnification of the animal polar region of the fertilized egg, showing the first and second polar bodies (arrowhead). D. Two-cell stage embryo. Lateral view. E. Regular four-cell stage embryo. Animal polar view. F. Irregular four-cell stage embryo. Animal polar view. Note the irregular arrangement of the blastomeres. G. Regular eight-cell stage embryo. Lateral view. H. Irregular eight-cell stage embryo. Lateral view. I. Blastula having a narrow blastocoel (BC). J. Swimming blastula. Primary mesenchyme (P) migrates to the blastocoel. The hyaline layer is thickest at the posterior pole (arrowhead). K. Swimming blastula slightly compressed to show vacuoles in the animal polar wall (arrowheads). Primary mesenchyme has occupied almost the whole cavity of the blastocoel. L. Gastrula. Arrowhead indicates the archenteron.

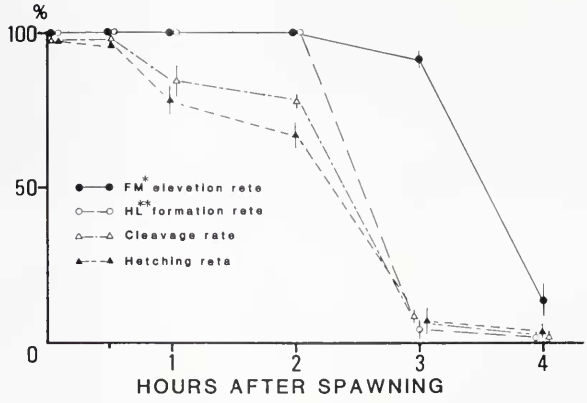

FIGURE 3. Relationship between the ability of the egg to develop normally and the time of insemination after the spawning at 23°C. Measurement was made three times and about 100 eggs were examined in each measurement. Mean ± standard deviation. FM*, Fertilization membrane; HL**, Hyaline layer.

cell lineage. Successive divisions occur at approximately 40-min intervals, producing a spherical blastula with a narrow blastocoel (Fig. 2I).

The embryos hatch in the blastula stage. Usually the swimming blastulae gather near the water surface. A newly hatched, swimming blastula is still surrounded by the

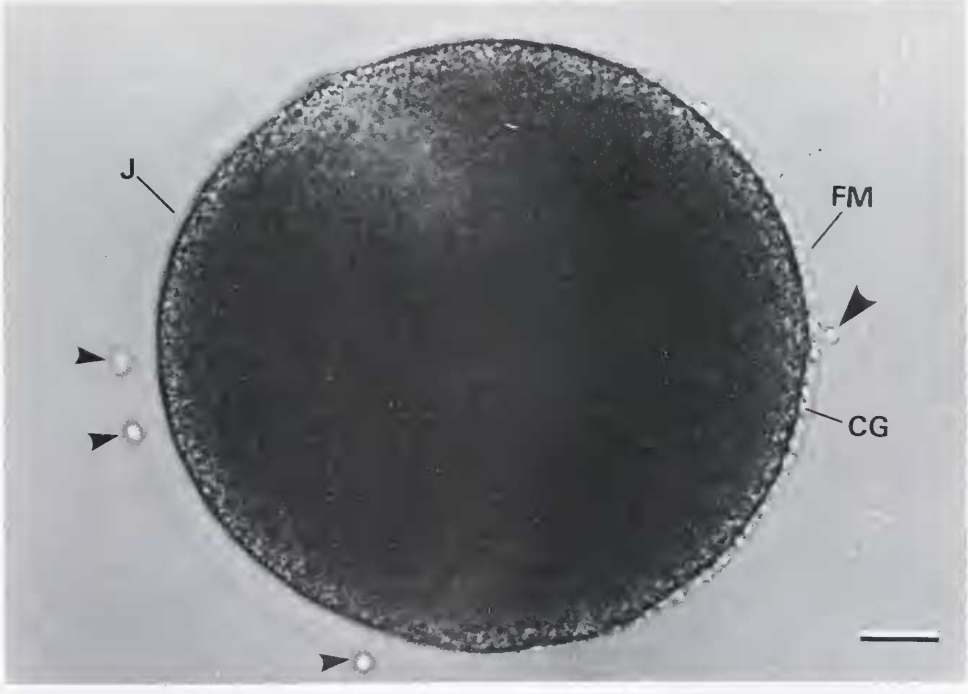

FIGURE 4. An egg during fertilization. The spermatozoon entering the egg is indicated by a large arrowhead. The other spermatozoa (small arrowheads) are attached to the jelly surface and are unable to enter the jelly coat (J). The discharged cortical granules (CG) and developing fertilization membrane (FM) are also seen. Note that the jelly coat begins to dissolve at the region of sperm entry. Scale, 10 μm.

TABLE I

Timetable for the embryonic development of Amphipholis kochii *egg cultured at 23°C and 15°C*

Stage	23°C	15°C	Q_{10}
First polar body	15 (min)	—	—
Second polar body	30	—	—
Two-cell	1.5 (h)	2 (h)	1.43
Four-cell	2⅙	3	1.50
Eight-cell	2⅚	4	1.54
Blastula	7–8	10–12	1.32–1.96
Hatching	8.5	13–14	1.70–1.87
Gastrula	13–17	1 (day)	1.53–2.15
Spicule formation	21	1.5	1.96
Archenteron differentiation	1.1 (day)	1.8	1.85
Coelomic pouch formation	1.3	2	1.71
Two-armed ophiopluteus	1.7	2	1.22
Left posterior coelomic pouch formation	1.7	3	2.03
Four-armed ophiopluteus	2	4	2.38
Right posterior coelomic pouch formation	2.5	6.5	2.98
Six-armed ophiopluteus	4.5	8.5	2.21
Eight-armed ophiopluteus	6	—	—
Hydrocoel formation	6.5	10.5	1.82
Hydrocoel five-lobed	8.5	—	—
Metamorphosis (incipient)	12	—	—

thick hyaline layer which is thickest at the posterior pole (Fig. 2J). Cells in the vegetal polar wall migrate into the blastocoel and occupy almost the whole cavity (Figs. 2J, K; 7A). These are probably the primary mesenchymal cells. Concurrently with the

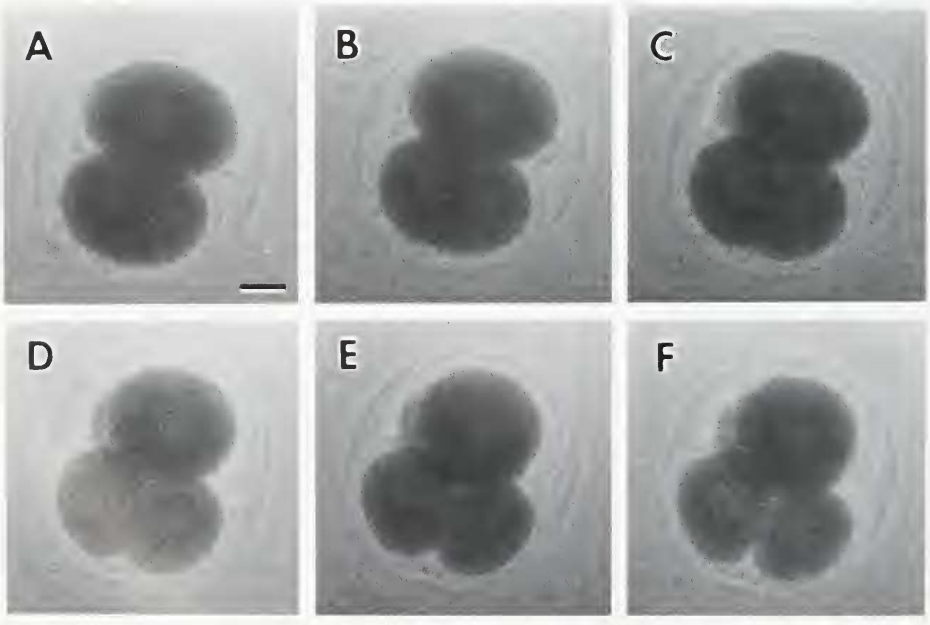

FIGURE 5. The second cleavage as observed by time-lapse cinematography. The second cleavage planes in two blastomeres are perpendicular to each other, producing an irregular arrangement of the blastomeres. Photographs were taken from 1 min intervals in the film. Scale, 20 μm.

cellular migration, the spherical swimming blastula becomes dorso-ventrally flattened and ellipsoidal in shape (Figs. 2J, K). The many vacuoles in the animal polar wall (Figs. 2K, 7A) may serve as a floating device, as suggested by Narasimhamurti (1933).

Gastrulation was achieved by an invagination of the wall cells at the vegetal pole (Figs. 2L, 7B, C). During gastrulation, the embryo becomes more dorso-ventrally flattened, taking a shield-like shape (Fig. 2L). At this stage, spicules begin to form in the primary mesenchyme (Fig. 6A), and take a tetraradiate shape (Fig. 6B).

Later development and metamorphosis

The early two-armed ophiopluteus is helmet shaped due to the appearance of the postero-lateral arms (Fig. 6C). Brownish red pigments are first observed in this larva, especially at the tip of the larval arms. At this stage, the archenteron differentiates into an intestine, stomach, and esophagus (Fig. 6C). The right and left coelomic pouches, which are derived from two pockets of the archenteron (Fig. 7C), are found near the constricted region between the stomach and esophagus (Fig. 6C). The antero-lateral arms are the second pair formed (Fig. 6D). The left posterior coelom is detected in the early four-armed ophiopluteus (Fig. 6D). It is uncertain whether the posterior coelom is formed by segregation of the original coelomic pouch. The stomach enlarges during the four-armed stage, after which the larva begins to eat diatoms. At the age of 4.5 days, the larva produces the post oral arms, resulting in a six-armed ophiopluteus (Fig. 6E). Finally, in the 6-day-old larva, the fourth arms, the postero-dorsal arms, are formed (Fig. 6G).

The eight-armed ophiopluteus reaches 350 μm in length along the antero-posterior axis, excluding the length of the antero-lateral arms. The larval skeletal system of *A. kochii* is very simple, without having any of the accessory rods described in other ophioplutei (Fig. 8; *cf.*, Mortensen, 1921). In the eight-armed larva, the left posterior coelom divides into a hydrocoel and a somatocoel (Fig. 7D). The left anterior coelom develops as an axocoel. The hydrocoel expands anteriorly along the stomach and differentiates into five lobes at the side of the esophagus (Figs. 6H, 7E). Formation of the right posterior coelom occurs in a later stage than that of the left posterior coelom (Table I; Figs. 6F, 7D). Further development of these organs is uncertain in this study.

Metamorphosis of the ophiopluteus is gradual. During the metamorphosis of *A. kochii*, the spicule of the adult skeletal system forms a triradiate shape which continues to branch, finally forming a network of spicules (Fig. 8). The anterior part of the larva shrinks and the larval arms degenerate except for the postero-lateral ones (Fig. 6I). The five-lobed hydrocoel surrounds the esophagus before forming the pentaradial water canal system of the adult brittle-star. As a result of these modifications, the anterior part of the larva develops into a young brittle-star which bears several podia (Fig. 6I). At the completion of metamorphosis, the last larval arms, the postero-lateral arms, degenerate.

Discussion

In the ovary of *A. kochii*, we were unable to find secondary oocytes or ova even during the breeding season (Iwata and Yamashita, 1982). Since the spawned eggs remain in the metaphase of the first meiotic division, oocyte maturation, including the breakdown of the germinal vesicle and the entrance into the meiotic division, occurs immediately before spawning. In fact, the ovarian oocytes show the germinal vesicle breakdown after the temperature shock (unpub. obs.). A similar situation for

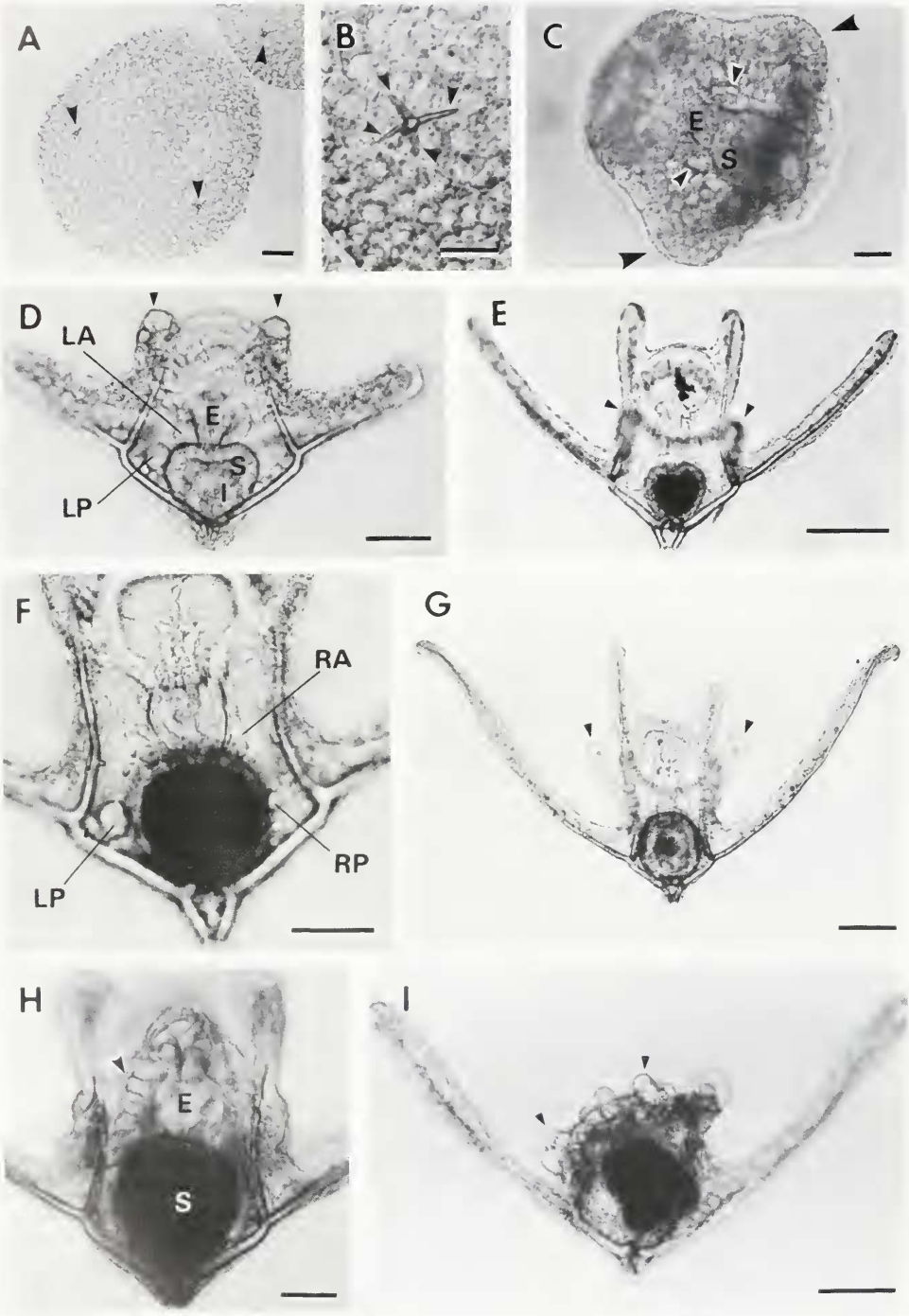

FIGURE 6. Gastrula and ophioplutei from the early two-armed to metamorphosis stage. Scale, 10 μm (B), 20 μm (A, C), 50 μm (D, F, H), 100 μm (E, G, I). A. Gastrula compressed to show the tetraradiate spicules (arrowheads). B. High magnification of the tetraradiate spicule showing the rudiments of the four larval skeletal rods (arrowheads). C. Early two-armed ophiopluteus. Large arrowheads indicate the rudiments of the postero-lateral arms. Apparent coelomic pouches (small arrowheads) are found between the stomach (S) and esophagus (E). The intestine is out of focus. Ventral view. D. Early four-armed ophiopluteus. The

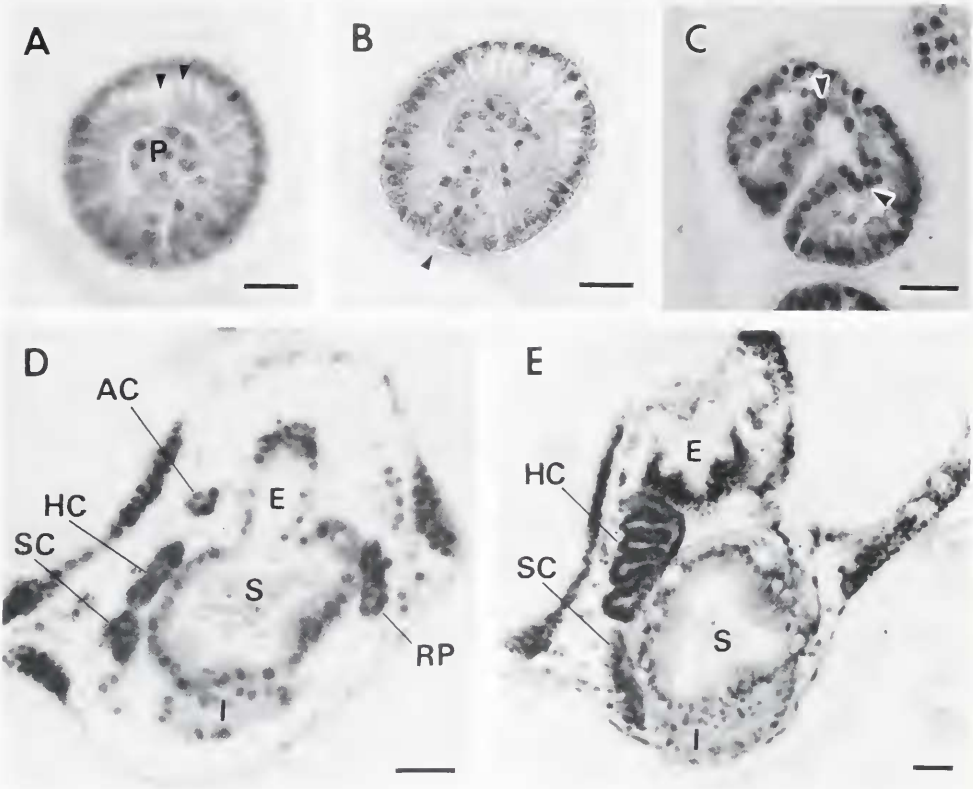

FIGURE 7. Histological longitudinal sections of the embryos and larvae. Scale, 20 μm. A. Blastula. Primary mesenchyme (P) has occupied the blastocoel. Arrowheads show the vacuoles in the animal polar wall. B. Early gastrula, showing invagination at the vegetal pole (arrowhead). C. Late gastrula. Archenteron has formed. Arrowheads point out two pockets in the archenteron, which are the origin of the coelomic pouches. D. Eight-armed ophiopluteus. The left posterior coelom has divided into hydrocoel (HC) and somatocoel (SC). AC, axocoel; E, esophagus; I, intestine, RP, right posterior coelom; S, stomach. E. Late eight-armed ophiopluteus. The hydrocoel (HC) has developed along the esophagus (E) and become a five-lobed shape. The somatocoel (SG) has grown at the side of the stomach (S). I, intestine.

oocyte maturation has been reported in the asteroids, *Asterias amurensis* and *Asterina pectinifera*, in which oocyte maturation is induced by the neurosecretory system immediately before spawning (*cf.*, Kanatani, 1973).

It is notable that the spawning of the asteroids, induced by a gonad stimulating substance (GSS) from the radial nerve, also has a time lag of similar duration to the period observed after the temperature shock of *A. kochii* (Kanatani and Ohguri, 1966). This similarity implies that the temperture shock given for the spawning of *A. kochii*

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antero-lateral arms are indicated by arrowheads. The left anterior (LA) and posterior (LP) coeloms are seen. The larval digestive system has developed into the esophagus (E), stomach (S), and intestine (I). Dorsal view. E. Six-armed ophiopluteus. The third arms, post oral arms (arrowheads), are seen. Ventral view. F. Six-armed ophiopluteus, in which the right anterior (RA) and posterior (RP) coeloms are seen. The left posterior coelom is also seen (LP). Dorsal view. G. Eight-armed ophiopluteus. The postero-dorsal arms (arrowheads) are prominent. Dorsal view. H. Ophiopluteus with five-lobed hydrocoel (arrowhead). The esophagus (E) and stomach (S) are also indicated. I. Ophiopluteus during metamorphosis. Arrowheads indicate the podia of a young brittle-star. Dorsal view.

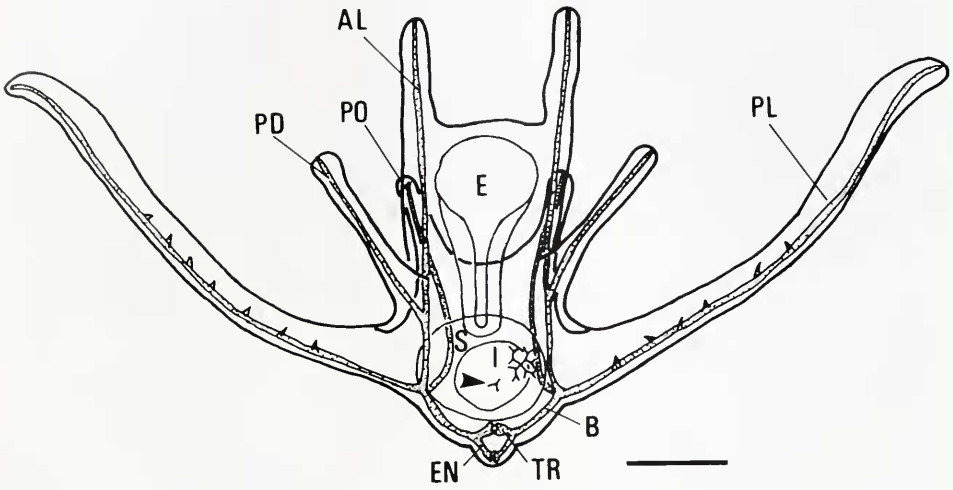

FIGURE 8. Eight-armed ophiopluteus, showing a larval skeletal system. For simplicity, coeloms and other small structures were omitted. Dorsal view. Arrowheads indicate the spicules of adult skeletal system. AL, antero-lateral rod; B, body rod; E, esophagus; EN, end rod; I, intestine; PD, postero-dorsal rod; PL, postero-lateral rod; PO, post oral rod; S, stomach; TR, transverse rod. Scale, 100 μm.

directly induces the nerve to release the GSS. However, it is still uncertain whether oocyte maturation and the spawning of the ophiuroids are controlled by the neurosecretory system as in the asteroids, although Fontaine (1962) has shown the presence of the neurosecretory cells in the radial nerve of the ophiuroid. Therefore, further experimental studies, such as *in vitro* maturation of ophiuroid oocytes, are necessary to clarify the mechanism controlling oocyte maturation and the spawning of ophiuroids.

The irregular arrangement of the blastomeres observed in the early cleavage stages of *A. kochii* egg has also been described in such ophiuroids as *Ophiopholis aculeata, Ophiothrix fragilis,* and *Ophiura albida.* It has been suggested that the irregularity of the blastomere arrangement is induced by the pressure exerted by the hyaline layer (Olsen, 1942). The fertilization membrane- and hyaline layer-free embryos (denuded embryos) of *A. kochii* obtained by treating with Ca-Mg-free sea water shows a mass of the blastomeres, which is easily scattered (unpub. obs.), as is the case of the denuded embryo of the asteroid (Dan-Sohkawa, 1976). It is therefore apparent that the hyaline layer plays an important role in the arrangement of the blastomeres in the ophiuroid embryo. However, the present study also demonstrates that the irregular arrangement of the blastomeres is caused not only by rearrangement of the blastomeres after regular radial cleavage, which is probably induced by the thick hyaline layer, but by an intrinsic, irregular cleavage plane in the blastomeres. An intrinsic irregular cleavage in the early embryos has been also found in many mammals (Gulyas, 1975). Moreover, various kinds of cleavage patterns, *e.g.,* irregular arrangement of blastomeres as observed in this study, or fusion of blastomeres after cleavage, have been reported in the asteroid embryos which develop quite normally (Teshirogi and Ishida, 1978). The irregular arrangement of blastomeres during the early cleavage may be an unusual pattern in animal eggs, but further detailed observations on the early cleavage of additional species are necessary.

As a rule, the larval spicules of the ophioplutei take a triradiate form (*cf.,* Mortensen, 1921). Therefore, the tetraradiate spicule present in *A. kochii* is remarkable when

compared with most other reported ophioplutei. The triradiate spicule in other ophioplutei consists of the antero-lateral, postero-lateral, and body rods, and the post oral rods are formed in the later stage of embryonic development. In *A. kochii,* however, the post oral rod is formed at the same stage as that of the earlier three rods. This precocious formation of the post oral rod gives the spicule of *A. kochii* a tetraradiate shape and may be related to a relatively rapid development of *A. kochii* as compared with other typical ophioplutei (*cf.,* Hendler, 1975). The simple skeletal system of this larva also seems to reflect the relatively rapid embryonic development of this species.

Other *Amphipholis* species hitherto studies are all littoral and viviparous (Mortensen, 1920; Fell, 1946; Oguro *et al.,* 1982; see also Hendler, 1975). The present study, however, reveals a littoral *Amphipholis* species with rapid, planktotrophic development. This confirms the notion that the developmental pattern in ophiuroids is related to ecological rather than phylogenetic factors (Fell, 1966; Nichols, 1969; Hendler, 1975).

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